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Specific Lectin Binding to Beta1 Integrin and Fibronectin on the Apical Membrane of Madin-Darby Canine Kidney Cells

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Abstract. Although lectins have previously been used to identify specific cell types in the kidney and various other tissues, the proteins labeled were not identified. We hypothesized that fluorescently labeled lectins could provide a useful tool for direct labeling of membrane-associated glycoproteins. Protein fractions from Madin-Darby canine kidney (MDCK) cells were exposed to a panel of 16 fluorescently labeled lectins to identify suitable lectin-protein pairs. Peanut agglutinin (PNA) selectively bound a 220–240 kDa O-linked glycoprotein with a slightly acidic isoelectric point, while *Sambucus nigra* agglutinin (SNA) labeled a 130 kDa glycoprotein with a highly acidic isoelectric point. Both proteins were readily labeled by lectins applied to the apical surface of living confluent cells. The proteins were isolated by lectin affinity columns and identified by mass spectrometry. Peptides from the PNA-binding protein shared molecular weight and amino acid composition with fibronectin. Fragments of the SNA-binding protein showed amino-acid identity with peptides from beta1 integrin. The identities of these proteins were validated by Western blotting. Binding of PNA to a 220 kDa protein was inhibited by an anti-fibronectin antibody, and binding of a 130 kDa protein by SNA was diminished by an anti-beta1 integrin antibody. We conclude that PNA and SNA can be used as specific markers for fibronectin and beta1 integrin, respectively, in MDCK cells.

Key words: Fluorescence — Mass spectrometry — Glycoprotein — Lectin

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Introduction

Hans H. Ussing remained a remote, legendary figure in our laboratory until it was announced in 1985 that he soon would be coming to the NIH as a Fogarty International Scholar-in-Residence. The prospect of working closely with the father figure of epithelial physiology was both exciting and intimidating. His decades of study of sodium transport mechanisms across frog skin had served to educate and instruct us all in physiology and the scientific method, and we had a vision of a man who was larger than life. What appeared instead of a legend living on his laurels was a vital, dedicated scientist with an extraordinary enthusiasm for research and a delightfully open and friendly demeanor. When the plan for new experiments was discussed, he focused his considerable intellectual energies on the transport of anions in epithelia, particularly on the question of the role of mitochondria-rich cells in transepithelial anion absorption. What resulted were three papers that served to characterize the role of mitochondria-rich cells in ion transport across the skin of frogs and toads and to draw attention to chloride channels in the apical membrane of these cells (Foskett & Ussing, 1986; Spring & Ussing, 1986; Larsen, Ussing & Spring, 1987). Hans Ussing became a lifelong friend, colleague and confidant whose delight in the mysteries of the biological world endured until his final days. Although his status in the field continued to grow over the decades, his attitude was always tempered by his modesty. An interaction with a new postdoctoral fellow illustrates this when he was asked "Ussing of the Ussing chamber?" and replied, "Yes, people are always surprised that I am a living person." Alas, we no longer have him as a living person, but continue to revere him.

Hans Ussing's interest in the role of mitochondria-rich cells in epithelial transport and in the details of the apical cell membrane constitutes the foundation upon which the present investigation has been constructed. The mitochondria-rich cell of toad and frog skin is analogous to the intercalated cell of the renal collecting duct. This cell, like the mitochondria-rich cell, is thought to be involved both in acid-base and anion transport. The MDCK (Madin-Darby canine kidney) cell line was derived from renal collecting duct and contains principal and intercalated cells. One method for discriminating between the two cell types is to look for differences in their relative affinity for binding lectins to the apical cell membrane. Peanut agglutinin (PNA), in particular, has been frequently used to identify intercalated cells because of its heavy apical labeling (Truong et al., 1988; Schuster et al., 1991; Minuth et al., 1993; Constantinescu, Silver & Satlin, 1997), although the binding site for the lectin has never been identified. Remarkably, although a wide variety of lectins are utilized to separate cell types or proteins, the proteins or lipids to which the lectins attach rarely have been identified. This may be due, in part, to the fact that widely used lectins, such as wheat germ agglutinin, bind to a variety of proteins and lipids that share common glycosylation moieties. Lectins have several advantages as markers of membrane proteins; they are extremely specific, they label living cells, they are nontoxic if not internalized, and many are available with fluorescent labels. They could prove useful as a tool for monitoring the number and distribution of proteins or lipids in living cell membranes under a variety of experimental conditions. We reasoned that a search of all available groups of fluorescently labeled lectins might reveal members that labeled only a single glycosylated protein or glycolipid in the apical membrane of MDCK cells. Therefore, we isolated the proteins associated with the plasma membrane from MDCK cells on both one- and two-dimensional gels, determined the lectin-binding patern of these proteins for virtually all commercially available lectin groups that were fluorescently labeled. We show that two lectins, PNA and elderberry bark agglutinin (*Sambucus nigra* agglutinin, SNA) each bind to a single protein associated with the apical membrane of MDCK cells. Identification of these proteins was accomplished by mass spectrometry and it is shown that PNA binds to fibronectin and SNA to β1-integrin.

Materials & Methods

Cell Culture

Low-resistance MDCK cells of passage 62 to 72 (American Type Culture Collection Rockville, MD) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and 2 mM glutamine without antibiotics or phenol red. Cells were kept at 37°C in 5% CO_2/95% atmospheric air on 10-cm diameter plastic Petri dishes and were used after 5 to 23 days. Culture medium was changed every 2–3 days.

Protein Fractionation

Crude plasma membrane protein fractions were isolated by a method modified from Moran, Handler & Turner (1982). Cells were lysed in a hypoosmolar buffer containing (in mM) 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 15 phosphate, 15 Na^+, 30 mannose, 5 mg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin (pH 7.0). After cell homogenization for 3×15 seconds at full speed (OMNI-TH homogenizer fitted with a micro-saw tooth generator), the lysate was separated into three fractions by centrifugation. After 10 minutes at $4{,}500 \times g$, the pellet was collected (*P*1, heavy organellar material and unbroken cells) and the supernatant (*S*1) was centrifuged for an additional 10 minutes at $17{,}500 \times g$. Both the pellet and the supernatant were collected (*P*2, plasma membrane fraction, and *S*2, water soluble proteins). This procedure was performed at 2–5°C. The protein content was determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Protein samples were adjusted to 1.5% (w/v) sodium dodecyl sulphate (SDS), 38.9 mM 1, 4-dithiothreitol (DTT), 6 (v/v) glycerol, 10 mM tris (hydroxymethyl) aminomethane (Tris), pH 6.8 and added bromophenol blue. The samples were heated to 60°C for 10 minutes and kept at −80°C until use.

The cell fractionation was validated by immunoblotting and immunocytochemistry of confluent MDCK cells. A mouse monoclonal anti-Na^+/K^+ ATPase α-1 antibody (Upstate Biotechnology, Lake Placid, NY) was applied as a basolateral marker and an affinity purified rabbit anti-vasopressin-regulated urea transporter antibody (L403, kindly provide by Mark Knepper, NHLBI, NIH, Bethesda, MD) was used as an apical surface marker.

SDS-PAGE and Electrotransfer

After heating to 37°C for 5 minutes, 5 μg of protein was loaded into 7.5 or 10% polyacrylamide Ready-Gels (BioRad, Hercules, CA), run for 5 min at 100 V (stacking) and separated for 35 min at 200 V using an electrode buffer of 25 mM Tris, 192 mM glycine, 0.1% SDS (Laemmli, 1970) supplemented with 10 μM NaN_3. Molecular-weight markers were from BioRad. Proteins were either directly stained (Coomassie Blue R-250 or Silver Stain Plus according to manufacturer, BioRad) or electro-transferred for 1 hour at 250 mA onto 0.45 μm pore size nitrocellulose membranes (BioRad).

Lectin Overlay

Nitrocellulose membranes were blocked with 10 mg/ml bovine serum albumin (BSA) for 30 minutes in Tris-buffered salt solution (TBSS, in mM: 150 NaCl, 10 Tris, 1 $CaCl_2$, 1 $MgCl_2$, pH 7.0). After washing in TBS, the membranes were exposed for 30 minutes to 10 μg/ml lectin conjugated to fluorescein or fluorescein-5-isothiocyanate (FITC). Excess dye was removed by 6 washing steps in TBSS. Membrane fluorescence and gel protein stain were detected on a Molecular Imager FX (BioRad).

FITC-conjugated PNA was purchased from Sigma-Aldrich (St. Louis, MO), and an SNA-fluorescein conjugate was from Vector Laboratories (Burlingame, CA). Neuraminidase and β-galactosidase were obtained from Sigma-Aldrich and O-glycosidase was from Roche (Nutley, NJ).

Lectin-Glycolipid Binding Assay

Plasma membrane fractions of MDCK cells were incubated with 50 μg/ml proteinase K or 50 μg/ml of the protease inhibitor leupeptin 37°C overnight. Samples were exposed to 10 mg/ml fluorescent lectins, washed twice and the fluorescence detected by spectrofluorometry. Emission scans from wavelength 500–550 nm were recorded at excitation 488 nm on a FluoroMax 2 spectrofluorometer (ISA, Jobin Yvon-Spex, Edison, NJ). Protein-specific labeling was calculated at emission wavelength 520 nm for PNA-FITC fluorescence and 508 nm for SNA-fluorescein.

Lectin Labeling of Apical Membranes

Confluent layers of living MDCK cells were washed three times in HEPES buffer before apical exposure to 10 μg/ml fluorescently labeled lectin for 5 min at 37°C. After washing three times with HEPES buffer, sugar groups were oxidized for 10 minutes by 2 mM $NaIO_4$ in HEPES buffer. Cells were exposed to 500 μM of the chemical cross linker *p*-azidobenzoyl hydrazide (ABH) for 10 min. ABH was UV-activated for 1 minute to induce covalent linkage of lectin and the bound membrane glycoprotein. After extensive washing, the cells were lysed, homogenized, fractionated and proteins used for SDS-PAGE.

Two-Dimensional Gel Electrophoresis

Membrane protein fractions were solubilized in 2% 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulphonate (CHAPS) and 1% TritonX-100, heated to 60°C for 10 minutes and loaded onto pre-electrophoresed capillary gels for isoelectric focusing, creating a gradient of pH ~3–10. Up to 70 μg protein were loaded per sample and run for 6 or 16 hours at 650 V at 5°C. The gels were then incubated with SDS and bromophenol blue for 15 minutes and placed on a 7.5% separation gel (BioRad) and run 35 min at 200 V. Hereafter, the proteins were either stained or electrotransferred to nitrocellulose membranes for lectin overlay as described above.

Affinity Isolation of Lectin-binding Proteins

Whole-cell homogenates or P2 protein fractions were solubilized in 2% CHAPS (TBS) and exposed to 1ml lectin-coated agarose-beads for 10 minutes at 37°C in a total volume of 14 ml TBS. Agarose bead-conjugated PNA was purchased from Sigma-Aldrich and SNA-agarose beads were obtained from Vector Laboratories. After washing five times in 10 ml TBS, proteins were eluted by exposure to 200 mM of the competing sugar for 10 minutes in a volume of 100 μl (galactose for PNA, lactose for SNA). The isolates were heated and separated by 7.5% or 10% SDS-PAGE and stained with Coomassie blue as described above.

In-Gel Digestion, Mass Spectrometry and Data Base Search

In-gel digestion of SDS-PAGE-separated proteins was carried out as described by the Association of Biomolecular Resource Facilities (1997). The protein bands were excised, washed, and in-gel digested using modified porcine trypsin (Promega, Madison, WI). The resulting peptides were extracted in two steps, partially dried, redissolved in 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich); salts were removed using C18-ZipTip® (Millipore, Bedford, MA) solid-phase extractions into 10 μl of 1:1 0.1% TFA:acetonitrile.

The extracted peptides were analyzed by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS). The extracts were partially dried and resuspended in 9 μl of 0.1% TFA. An aliquot of this resuspended sample, 2–5 μl, was injected onto a packed capillary gradient MAGIC LC system (Michrom Bioresources, Auburn, CA) operated at 400 nl/min using a Michrom Magic constant pressure splitter. Samples were separated using a 10-min linear gradient, 2%–85% B (*A*, 5% acetonitrile in water with 0.5% acetic acid and 0.005% TFA; *B*, 80% acetonitrile in water with 0.5% acetic acid, 0.005% TFA) with a Vydac C18, 5-μm particle, 300-Å pore packing material. Columns, approximately 5 cm in length, were packed by hand into a 75-μm I.D. fused silica capillary (PicoFrit® , New Objective, Woburn, MA). The LC effluent was electrosprayed directly into the sampling orifice of an LCQ DECA spectrometer (Thermo Finnigan, SanJose, CA) using an adaptation of the microscale electrospray interface (Davis et al., 1995). The LCQ DECA was operated in a mode that automatically generated MS-MS spectra of the four most intense peaks present in any single scan of the ion trap that exceeded a pre-set threshold. Peptide partial internal sequence data produced by the LC-MS/MS experiments were subjected to analysis using both SEQUEST (Eng, McCormack & Yates, 1994) and MASCOT (Perkins et al., 1999) programs employing the mammalian portions of non-redundant data bases.

Western Blotting

After SDS-PAGE and electrotransfer, the nitrocellulose membranes were blocked in 5% (wt/vol) nonfat dry milk in PBS (150 mM NaH_2PO_4 and 0.05% Tween 20, pH 7.5) for 1 hour at 21°C and incubated overnight at 5°C with primary antibodies in PBS with 0.1% (wt/vol) BSA. Primary antibodies were from Chemicon (Temecula, CA): mouse monoclonal anti-fibronectin antibody (MAB 1940 and MAB 1926) and mouse monoclonal anti-beta1 integrin antibody (MAB 2000) or rabbit anti-beta1 integrin antibody (AB 1952). After washing, the membranes were incubated with FITC-conjugated secondary antibody in PBS with 0.1% (wt/vol) BSA for 1 hour at 21°C. Antibody binding was visualised on a Molecular Imager FX (BioRad).

Results

Selection of Membrane Protein-binding Lectins

Effective isolation and identification of membrane-associated proteins by lectin binding required the selection of suitable lectins that bound only one or few glycoproteins in one-dimensional gel electrophoresis. Initially, a panel of 16 fluorescently labeled lectins was screened by SDS-PAGE and lectin overlay using protein fractions from MDCK cells (Table 1) . Although eight lectins labeled two or fewer bands on the overlays, strong fluorescence signals from both the overlay and the apical cell surface occurred only for PNA and SNA.

PNA bound to glycoproteins of ~220 and ~240 kDa from MDCK cells, as illustrated in Fig. 1*A*. The labeling was most intense for proteins from the 17,500 × *g* pellet (the plasma membrane fraction, P2) although a low level of fluorescence was detected from the proteins of the 4,500 × *g* pellet (P1) as well. PNA-FITC signal was absent from the proteins of

Table 1. Lectin Panel

Lectin and (Common name)	Apical cellular fluorescence	Bright overlay fluorescence	Number of bands ⩽ 2
***Arachis hypogea* lectin (Peanut agglutinin)**	+	+	+
Canavalia ensiformis lectin (Jack bean aggl., Con A)	+	+	–
Dolichos bliflorus agglutinin (Horse gram lectin)	(+)	–	+
Galanthus nivalis lectin (Snowdrop bulp lectin)		+	–
Glycine max agglutinin (Soybean agglutinin)	–	–	–
Griffornia simplicifolia lectin Ib4	(+)	–	+
Griffornia simplicifolia lectin II	–	–	+
Lens culinaris agglutinin (Lentil agglutinin)		+	–
Limulus polyphemus lectin (Horseshoe crab lectin)	–	–	+
Phaseolus vulgaris agglutinin (Kidney bean lectin)	–	–	+
Pisum sativum agglutinin (Garden pea lectin)		–	–
Ricinus communis agglutinin (Castor bean lectin)	–	–	+
***Sambucus nigra* agglutinin (Elderberry bark lectin)**	+	+	+
Triticum vulgaris lectin (Wheat germ agglutinin)	+	+	–
Triticum vulgaris lectin, succinylated	+	+	–
Ulex europaeus agglutinin I (Furze gorse lectin)	(+)	+	–

Apical cellular fluorescence intensity was determined by fluorescence microscopy. (+) indicates that only a subpopulation of cells were labeled. The number of bands was measured from lectin overlays. The two lectins that were positive in all three categories are shown in bold.

the 17,500 × *g* supernatant (*S*2). The results were independent of the maturation of the cells in the range 5 to 21 days after seeding. Figure 1*B* shows that the lectin binding was competed by incubation with 200 mM D(+)-galactose. PNA-labeling was also prevented by overnight incubation with 100 mU/ml O-glycosidase at 37°C (*not shown*).

SNA labeled glycoproteins of ~35 kDa and ~130 kDa from the P2 fraction (Fig. 1*C*). An ~80 kDa protein of the S2 fraction was also bound by SNA, whereas labeling was minimal in the *P*1 fraction. These results were obtained with cultured cells in the range 5 to 21 days after seeding. The labeling of the 130 kDa and the 80 kDa proteins was reduced by incubation with neuraminidase (Fig. 1*D*) or 200 mM α(+)-lactose (*not shown*), whereas labeling of the 35 kDa protein was unaffected by the treatment.

Analysis of Lectin Binding

The degree of lectin binding to cellular components other than proteins, such as glycolipids, was determined by protease treatment of whole-cell homogenate. The PNA-binding capacity was reduced by Proteinase K to 3.7% or 5.2% of the undigested control samples in repeated experiments. The SNA-binding capacity was reduced to 8.7% or 9.5% by Proteinase K (compared to control samples, $n = 2$). The decrease in lectin-binding capacity after protease treatment was paralleled by a decrease in total protein content to ~4%. Hence, the vast majority of the lectin binding is to glycoproteins and not to other cellular components.

To determine whether the PNA-and SNA-binding proteins were present on the apical surface of MDCK cells, fluorescently labeled lectins were applied prior to cell lysis. The migration of the labeled lectin, crosslinked to its specific apical protein, was detected directly from gels (Fig. 2). The PNA-protein complex migrated as a single ~270 kDa compound, whereas the SNA-protein complex migrated as ~270, 200 and 170 kDa complexes. The apparent MW of the complexes suggests that the 220–240 kDa protein migrated with a monomer or dimer of PNA and that the 130-kDa protein migrated bound to either tetramers, dimers or monomers of SNA.

Glycolytic analysis of the PNA-binding protein was performed for further characterization. Treating the PNA-agarose eluate overnight with O-glycosidase decreased the apparent molecular weight of the PNA-binding protein by 5–8 kDa. This shows that the target protein contains relevant O-glycosylation sites for PNA binding.

Isolation of Lectin-Binding Proteins

Figure 3*A* shows a Coomassie-stained gel after 2D-PAGE. The PNA- and SNA-binding proteins on the gel were identified after lectin overlay on membranes of proteins transferred from gels run in parallel (Fig. 3*B* and 3*C*). The PNA-binding proteins were both focused at slightly acidic pH. Isoelectric focusing for 16 hours was required for the PNA-binding proteins, but caused most of the 130 kDa and the 80 kDa SNA-binding proteins to migrate through the gel. Therefore, the electrophoresis was shortened to 6 hours to better focus these proteins at very low pH (Figure 3*D*). The 35-kDa SNA-binding protein focused near neutral pH.

Isolation of lectin-binding proteins on affinity columns was performed with both whole-cell homogenates and isolated P2 fractions. On a silver-stained gel, the eluted protein from the PNA-agarose column migrated with an apparent MW of 220 kDa and was

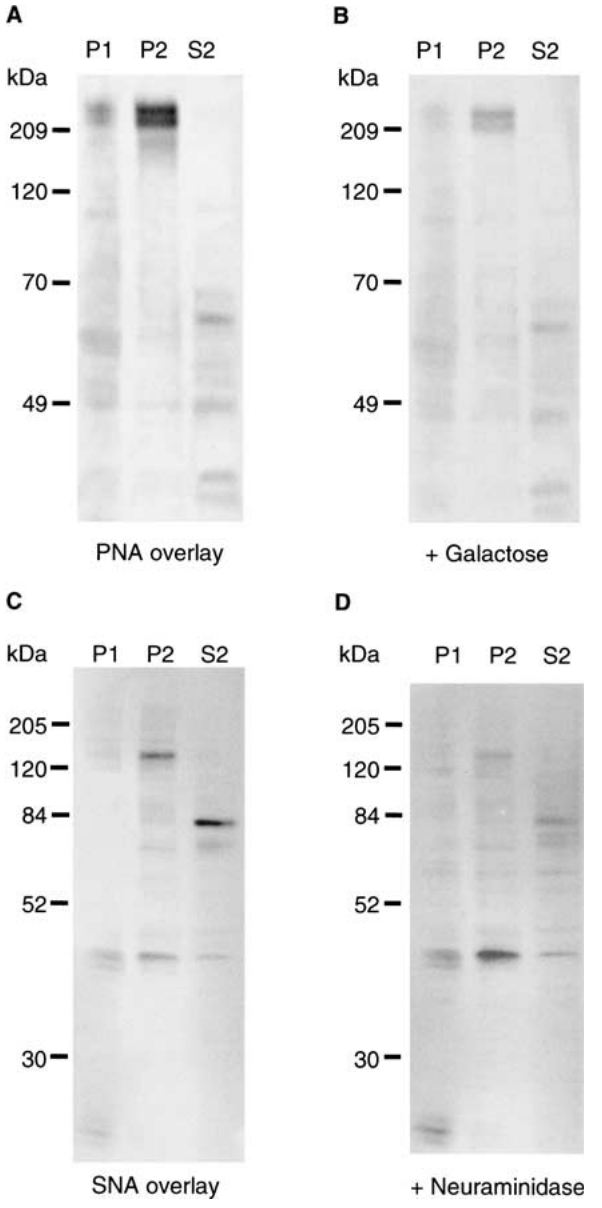

Fig. 1. Detection of glycoproteins binding PNA or SNA. The proteins of MDCK cells were fractionated and separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes for lectin overlay. *P*1 lanes represent the 4,500 × *g* pellet proteins, *P*2 lanes are the 17,500 × *g* pellets and S2 lanes the 17,500 × *g* supernatants. (*A*) PNA-FITC overlay after 7.5% SDS-PAGE and electrotransfer. (*B*) Elution of PNA binding with galactose. The same nitrocellulose membrane was incubated with 200 mM D(+)-galactose for 30 minutes at 21°C and re-analyzed. (*C*) SNA-fluorescein overlay after 10% SDS-PAGE and electrotransfer. (*D*) Reduction of SNA-fluorescein labeling by neuraminidase. The same nitrocellulose membrane was incubated with the sialic acid-specific glycosidase neuraminidase (5mg/ml) for 60 minutes at 37°C and re-analyzed.

capable of binding PNA-FITC (Fig. 4*A*). Several proteins were eluted from the SNA-agarose column using whole-cell homogenate, but only ~130 kDa, 80-kDa and 65-kDa proteins bound SNA-fluorescein

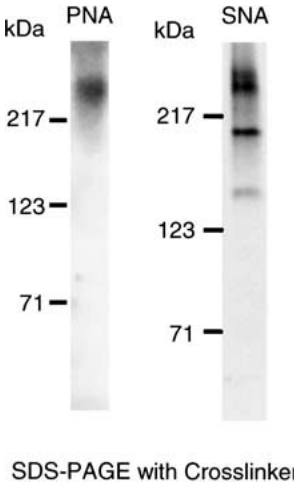

Fig 2. Prelabeling of glycoproteins associated with the apical membrane by fluorescent PNA or SNA. The apical surface of confluent living MDCK cells were exposed to 10 mg/ml lectin for 5 min at 37°C. After cross-linking the lectin-glycoprotein interaction with *p*-azidobenzoyl hydrazide (ABH), the cells were washed thoroughly, lysed and homogenized. The fluorescence of the glycoprotein-lectin complexes was visualized directly from the gels following separation by SDS-PAGE. The PNA-glycoprotein migrated as a single compound, while three bands were observed for the SNA-glycoprotein complex. This likely represents partial cleavage of one or more SNA subunits from the compound due to incomplete crosslinking within the lectin.

(Fig. 4*B*). Isolation with the SNA-agarose column using P2 fraction proteins yielded only 130-kDa and 35-kDa proteins (*not shown*).

Mass Spectrometric Identification of Isolated Proteins

Identification of the isolated proteins utilized the MASCOT probability-based searching approach with the LC-MS/MS output. The 240- and 220-kD PNA-binding proteins yielded results consistent with fibronectins. While the most significant hits were to non-canine fibronectins, identity to a canine partial fibronectin sequence was also found; 18% coverage of the sequence of this 58-kDa fragment was obtained. The 130-kDa SNA binding protein shared peptide composition with beta1 integrin from pig. In addition, a 240-kDa protein from the SNA column that did not bind SNA-fluorescein was identified as fibronectin. These results are summarized in Table 2.

Validation of Protein Identification

Antibodies against beta1 integrin and fibronectin were used to confirm the identity of the two lectin-binding proteins. As shown in Fig. 5*A* an anti-fibronectin antibody (MAB 1940) recognized a ~220 kDa protein of the plasma membrane fraction of MDCK cells. The same result was seen with the MAB 1926

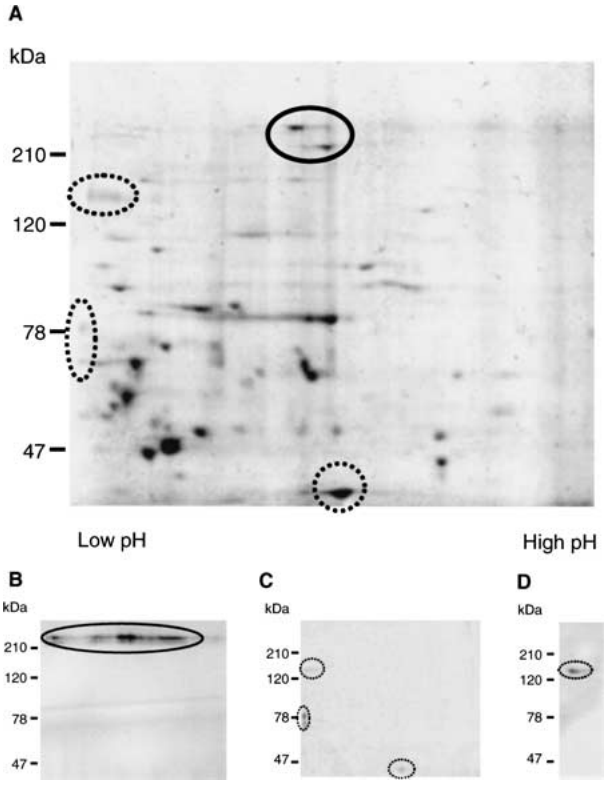

Fig. 3. Separation of MDCK cell proteins by 2-D PAGE. The proteins of MDCK cells were fractionated and only the 17,500 × g pellet was analyzed. (*A*) Coomassie-stained 10% acrylamide gel after 16 hours of isoelectric focusing (horizontal) and separation by molecular size (vertical). Full-line circle indicates PNA-binding protein, dotted-line circles are SNA-binding proteins identified by the lectin following lectin overlays. (*B*) PNA-fluorescein overlay of similar electro-transferred 2-D gel. Circle indicates PNA-binding protein. The apparent poorer protein focusing could be caused by a higher sensitivity of the lectin overlay than the coomassie stain. (*C*) SNA-fluorescein overlay of similar electro-transfered 2-D gel. Circles are SNA-binding proteins. Two of these proteins were partially run off the gel at acid pH. (*D*) SNA-fluorescein overlay of electro-transfered 2-D gel after only 6 hours of isoelectric focusing. Circle marks the better-focused SNA-binding protein.

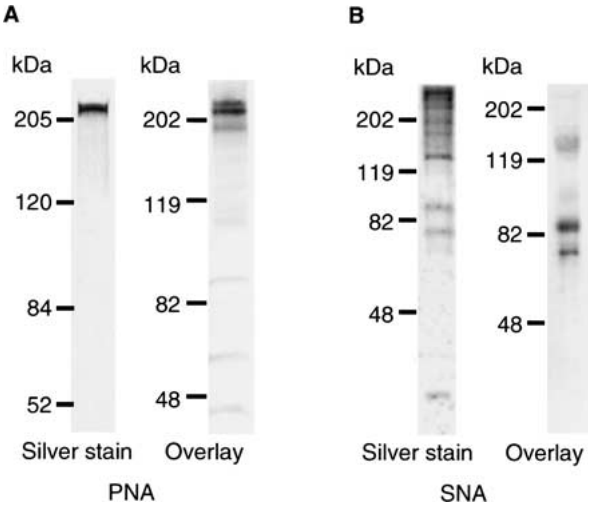

Fig. 4. Isolation of glycoproteins by lectin affinity column. Whole cell homogenates or 17,500 × *g* pellets were exposed to lectin-bound agarose beads and attached glycoproteins were eluted by the competing sugar. (*A*) Silver-stained 7.5% gel after SDS-PAGE with the PNA-agarose eluate and PNA-fluorescein overlay on similar gel after electrotransfer. (*B*) Silver-stained 10% gel after SDS-PAGE with the SNA-agarose eluate and SNA-fluorescein overlay on similar gel after electrotransfer.

antibody (*not shown*). The binding of PNA-FITC was competed by incubating the nitrocellulose membrane with the anti-fibronectin antibody (MAB 1940) prior to lectin overlay (Fig. 5*B*). Figure 5*C* illustrates that an anti-beta1 integrin antibody (MAB 2000) labeled a 130-kDa protein. The same result was seen with the MAB 1952 antibody (*not shown*). The binding of SNA-fluorescein was partially prevented by preincubation with anti-beta1 integrin antibody (MAB 2000, Fig. 5*D*). Binding of antibodies was likewise affected by preincubation by the relevant unlabeled lectin (*data not shown*).

Discussion

Tracking of cellular proteins is generally performed on either fixed preparations or cell fractionates. Live cell imaging of theses proteins is much more complicated and performed less frequently. Several approaches have been made, the most successful being joining the protein of interest to green fluorescent protein GFP or, more rarely, employment of antibodies directed against naturally folded extracellular epitopes of the target protein. In contrast to these methods, fluorescently labeled lectins have been used as a fast, simple and very reproducible method to label surface proteins on cultured cells even though the binding sites remained unknown (Gekle et al.,

Table 2. Summary of mass spectrometrical analysis

Sample	Protein ID	Accession number	MASCOT score*	Matching peptides	Coverage of protein (%)
PNA-240 kDa	Fibronectin precursor	2506872	540	15	8
PNA-220 kDa	Fibronectin precursor	2506872	197	4	3
SNA-140 kDa	Beta1 integrin	10336839	342	10	14
SNA-240 kDa	Fibronectin precursor	2506872	620	15	8

Proteins of the indicated molecular weight isolated by PNA and SNA columns were subjected to mass spectrometry.
* Score = 10(log *p*), where *p* = probability of identification. Score >44 indicates identity.

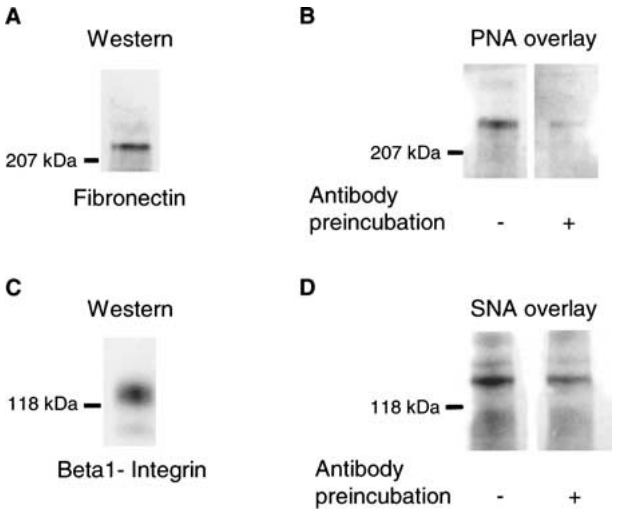

Fig. 5. Validation of proteins identified by mass-spectrometry. Proteins from the plasma membrane fraction were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes. (*A*) Western blotting performed with monoclonal anti-fibronectin antibodies and FITC-conjugated secondary antibody. (*B*) Competition of PNA-FITC labeling of membrane proteins from MDCK cells. PNA overlay was performed either without or with preincubation with anti-fibronectin antibody, as indicated. (*C*) Western blotting using an anti-beta1 integrin antibody and FITC-conjugated secondary antibody. (*D*) Competition of SNA-fluorescein-labeling of membrane proteins from MDCK cells. SNA overlay was performed either without or with preincubation with anti-beta1 integrin antibody, as indicated.

1994; Kovbasnjuk & Spring, 2000; Verkoelen et al., 2000). We hypothesized that some lectins would label only a single glycoprotein because of the vast range in glycan specificity of these molecules and, hence, provide a new tool for protein tracking in living cells.

As expected, most lectins in the tested panel showed affinity for several proteins of the plasma membrane fraction. However, the lectin screening did identify two candidates for further analysis: PNA, a lectin with high affinity for O-linked galactosyl (β-1,3) N-acetylgalactosamine, and SNA, that binds preferentially to a terminal sialic acid in (α-2,6) linkage to an N-acetylgalactosamine or galactose residue (Varki et al., 1999). Most frequently, PNA labeled both a 220- and a 240-kDa band on the lectin overlay. It seemed likely that these bands represented different forms of the same glycoprotein since they migrated with similar isoelectric points on 2D gels. This was confirmed by mass spectrometry. The two migration patterns likely represent alternative splicing of the encoding mRNA, or differences in phosphorylation or glycosylation of the protein. In some experiments, however, only the 220-kDa band appeared, probably because the higher molecular weight protein was not transferred to nitrocellulose membranes.

The specificity of the lectin-binding to the target glycoproteins was shown by sugar competition and by enzymatic cleavage of sugar groups. In fact, PNA binding was greatly reduced by the competing sugar and prevented by enzymatic cleavage of O-linked glycans. The binding of SNA to the 130-kDa plasma membrane protein was likewise reduced by lactose or neuraminidase (sialidase) treatment. In contrast, the 35-kDa SNA-binding protein of the plasma membrane fraction was resistant to both treatments and, thus, deemed to be nonspecific labeling. Thus, the binding of both PNA and SNA was sugar specific. It is noted that the 80 kDa SNA binding protein is unlikely to appear on the cell surface, since it was only found in the soluble protein fraction.

One objective of our study was to obtain evidence that the lectins could be useful as markers of apical glycoproteins in live cell-imaging studies. Therefore, it was of major interest to determine: 1) whether the lectin binding was restricted to glycoproteins, and 2) whether the target proteins were present on the apical surface of the cells. The possibility existed that a large fraction of the cellular labeling was due to binding to surface glycolipids, since these molecules share sugar residues with glycoproteins (Varki et al., 1999). Lectin-glycolipid interaction would not be revealed by SDS-PAGE but was readily ruled out by the virtually complete loss of lectin-binding capacity upon treatment with a proteolytic enzyme. The apical localization of the proteins was clearly demonstrated by the crosslinker experiments. Here, the lectin-glycoprotein interaction was preserved by covalent binding prior to cell lysis. Furthermore, the 35-kDa SNA-binding protein did not react with the lectin applied to the apical surface before crosslinking. This most likely indicates an abundant protein associated with the intracellular surface of the plasma membrane. Subsequent mass spectrometry showed that this protein was actin.

The PNA- and SNA-binding proteins were isolated from MDCK cells by lectin affinity columns in combination with SDS-PAGE. This method yielded larger quantities of the protein than did 2D gel electrophoresis. Fibronectin matched the PNA-binding protein of MDCK cells with respect to molecular weight (Boughton & Simpson, 1984) and is known to be an O-linked glycoprotein (Mandel et al., 1994). Furthermore, the slightly acidic isoelectric point of the PNA-binding protein from MDCK cells is consistent with earlier reports on fibronectin (Boughton & Simpson, 1984; Musante, Candiano & Ghiggeri, 1998). Some fibronectin forms have actually been shown to bind PNA directly (Trejdosiewicz et al., 1985). The molecular weight of beta1 integrin is equivalent to that of the SNA binding protein of MDCK cells and the isoelectric point is also strongly acidic (Veiga et al., 1995). Moreover, beta1 integrin is described as a sialated N-linked glycoprotein (Pretzlaff, Xue & Rowin, 2000; Veiga et al., 1995).

The protein identification was validated by lectin overlay and western blotting. First, both the anti-fibronectin antibody and PNA recognized proteins of identical molecular weights. The anti-beta1 integrin antibodies and SNA also both recognized proteins of identical molecular weights. Second, binding of PNA was inhibited by the anti-fibronectin antibody, implying that the antibody and the lectin bind the same protein on the nitrocellulose membrane. Likewise, SNA labeling was inhibited by anti-beta1 integrin, again suggesting that the lectins and antibody are recognizing the same protein. These findings very strongly support the conclusion that fibronectin and beta1 integrin are the PNA- and the SNA-binding proteins of MDCK cells, respectively.

Finding both of these proteins on the apical surface of MDCK cells could seem surprising at first. Fibronectin is normally secreted into the interstitial space of renal epithelia by various cell types and not to the lumen (Zuk, Bonventre & Matlin, 2001). On the other hand, secretion of fibronectin has been demonstrated from both basolateral and luminal plasma membranes of MDCK cells (Low et al., 1994), and seems important for the development of branching tubules by MDCK cells (Jiang et al., 1999; Jiang, Chuang & Tang, 2000). Furthermore, there are reports of luminal fibronectin in distal nephrons in a stone-forming rat model and in post-ischemic kidneys (Tsujihata et al., 2000; Zuk, Bonventre & Matlin, 2001) where the protein was suggested to be involved in the pathogenesis of the diseases. Nevertheless, the question arose whether the isolated fibronectin originated from the fetal calf serum of the culture medium or was secreted to the luminal side by the MDCK cells. The culture medium as the source of fibronectin was, however, readily ruled out since dog-specific amino acids and other non-bovine amino acids and peptides were identified by mass spectrometry. The fibronectin that labels with PNA on the apical surface of MDCK cells is very likely attached to an integrin, the cellular fibronectin receptor, since the PNA labeling occurs in the plasma membrane fraction of the cells and not with the soluble proteins.

The beta1 integrin subunit is described as a transmembrane glycoprotein involved in cell-cell contacts and cell-matrix interactions (Green, Mould & Humphries, 1998) and has been reported on both basolateral and apical aspects of MDCK cells (Schwimmer & Ojakian, 1995; Zuk & Matlin, 1996). Beta1 integrin is a basolateral membrane protein in collecting duct cells from normal rats but the polarized distribution was lost in regenerating epithelial cells after ischemic tubular damage (Romanov et al., 1997; Zuk et al., 1998). The protein has been suggested to play an important role in tubule formation during development and repair (Saelman, Keely & Santoro, 1995; Schwimmer & Ojakian, 1995; Zuk & Matlin, 1996). Hence the apical location of both fibronectin and beta1 integrin in MDCK cells is in agreement with previous investigations and has proven a highly relevant model for in vitro studies of kidney development and repair.

In conclusion, specific lectins can be used as technically simple, inexpensive markers for major cellular surface proteins. They can provide potentially powerful tools for tracking identified proteins in living cells. PNA and SNA are promising candidates for the study dynamics of fibronectin and beta1 integrin in living MDCK cells.

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